Is swelling of the secretory granule matrix the force that dilates the exocytotic fusion pore?

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ABSTRACT The swelling of the secretory granule matrix which follows fusion has been proposed as the driving force for the rapid expansion of the fusion pore necessary for exocytosis. To test this hypothesis, we have combined simultaneous measurements of secretory granule swelling using videomicroscopy with patch clamp measurements of the time course of the exocytotic fusion pore in mast cells from the beige mouse. We show that isotonic acidic histamine solutions are able to inhibit swelling of the secretory granule matrix both in purified secretory granules lysed by electroporation and in intact cells stimulated to exocytose by guanine nucleotides. In contrast to the inhibitory effects on granule swelling, the rate of expansion of the exocytotic fusion pore is unaffected. Therefore, as the rate of granule swelling was more than 20 times slower under these conditions, swelling of the secretory granule matrix due to water entry through the fusion pore cannot be the force responsible for the characteristic rapid expansion of the exocytotic fusion pore. We suggest that tension in the secretory granule membrane, which has recently been demonstrated in fused secretory granules, might be the force that drives the irreversible expansion of the fusion pore.

INTRODUCTION

Exocytosis begins with the formation of a narrow water-filled pore connecting the lumen of the secretory granule with the extracellular space. Patch clamp measurements in mast cells have revealed that the fusion pore opens abruptly and has an initial conductance in the range 80–250 pS. Because of these properties, the opening of the fusion pore has been compared to the opening of an ion channel (Breckenridge and Almers, 1987*a*; Almers, 1990; Spruce et al., 1990). The initial fusion pore, which is small compared to the fusion pores seen in electron micrographs (Chandler and Heuser, 1980; Ornberg and Reese, 1981; Chandler et al., 1983, 1989), can either collapse, causing a transient fusion event and leaving an intact granule inside the cell, or expand to a much larger pore size, that allows release of the granule contents into the extracellular medium (Breckenridge and Almers, 1987*a*; Alvarez de Toledo and Fernandez, 1988; Spruce et al., 1990).

The dilation of the fusion pore and release of the granule contents is associated with a rapid swelling of the granule matrix (Breckenridge and Almers, 1987*b*; Zimmerberg et al., 1987). In contrast, during transient fusion events or during periods of reversible fluctuations in the fusion pore conductance that occur before irreversible pore dilation, no swelling is observed (Breckenridge and Almers, 1987*b*; Zimmerberg et al., 1987). These observations have led to the proposition that swelling of the granule matrix provides the force necessary to drive the irreversible expansion of the fusion pore and subsequent release of secretory products (Zimmerberg et al., 1987; Breckenridge and Almers, 1987*b*; Merkle and Chandler, 1989; Chandler et al., 1989). In support of this hypothesis are electron microscopy studies of sea urchin egg cortical granule exocytosis showing that fusion pore widening is apparently arrested when matrix swelling and dispersal is inhibited by hyperosmotic solutions and dextran solutions (Chandler et al., 1989; Merkle and Chandler, 1989). Furthermore, the fusion pore that forms between erythrocytes and fibroblasts expressing the influenza virus hemagglutinin is relatively stable and expands slowly (Spruce et al., 1989). In this fusion system, where no known swelling is associated with fusion, the rate of pore expansion is several orders of magnitude slower than that observed in exocytotic fusion.

In this study, we have designed experiments to test the hypothesis that the expansion of the exocytotic fusion pore is caused by swelling of the secretory granule matrix. Using the patch clamp technique and a phase-sensitive detector, we have examined the time course of the formation of the exocytotic fusion pore in mast cells from the beige mouse, a mutant with giant secretory granules. We have found that when the rate of swelling of the granule matrix is inhibited by >20-fold, achieved by bathing the cells in an extracellular medium containing histamine at acidic pH, the expansion of the fusion

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pore is neither prevented nor slowed. Thus, swelling of the granule matrix due to water influx through the fusion pore cannot be the driving force for the expansion of the exocytotic fusion pore in mast cells.

MATERIALS AND METHODS

Cell preparation

Mast cells were prepared from adult beige (bg^j/bg^j) mice (Jackson Laboratories, Bar Harbor, ME) after a procedure described in detail elsewhere (Alvarez de Toledo and Fernandez, 1990). Briefly, mouse peritoneal mast cells were prepared by peritoneal lavage, plated onto glass bottom culture chambers and stored at 37°C under a 5% CO_2 atmosphere until use. The medium for this incubation contained (in millimolar): 136 NaCl; 10 Hepes; 0.8 NaOH; 0.9 $MgCl_2$; 1.8 $CaCl_2$; 45 $NaHCO_3$; 0.8 K_2HPO_4; 2.5 Glucose; 0.12 mg/ml streptomycin, and 0.64 mg/ml penicillin (pH 7.2). Patch clamp measurements were performed at 21–23°C. The standard extracellular medium was a modified Ringer's solution containing (in millimolar): 150 NaCl; 10 Hepes; 2.8 KOH; 1.5 NaOH; 1 $MgCl_2$; 2 $CaCl_2$, and 25 Glucose (310 mmol/kg; pH 7.25). The acidic histamine medium contained (in millimolar): 130 histamine hydrochloride; 1 $CaCl_2$; 1 $MgCl_2$, and 5 citrate (pH 4.2–4.5). The histamine concentration and pH of this solution is similar to those found inside the granule in vivo (Alter and Schwartz, 1989) and was shown to inhibit swelling of granule matrix (Villalon et al., 1990).

Isolated secretory granule preparation

Mast cells were separated from the other cells present in the peritoneal lavage (macrophages and small lymphocytes) by using a metrizamide gradient (Yurt et al., 1977). The mast cell preparation was >90% pure as indicated by toluidine blue staining. Isolated secretory granules were obtained by rupturing the cell membranes using a sonication procedure: 1 ml of purified beige mast cells was subjected to five pulses of a sonicator (model 45; Branson Sonic Power Co., Danbury, CT) at 25% power. Aliquots of this suspension were plated onto glass bottomed chambers. Most of the granules strongly attached to the glass substrate within 30 min. The presence of an intact lipid membrane was demonstrated by using $DiIC_6(3)$, a lipophilic fluorescent dye.

Electroporation of intact beige mast cell granules

Pores in the membrane of the granules were induced by applying a high-intensity electric field in the form of an exponentially decaying capacitor discharge (Sowers and Lieber, 1986). A detailed description of this technique will be described elsewhere. Briefly, two flat parallel metal plates (5-mm width) were positioned facing each other 2 mm apart in a chamber containing the isolated granules on the stage of the inverted microscope. The ends of the plates were connected to the anode and cathode of an electroporation apparatus (Gene Pulser; Bio-Rad Laboratories, Cambridge, MA). The electrical breakdown of the granule membrane was induced by applying a high-intensity electric field (~ 10 kV/cm with a time constant of 200 μs). Lysis of the granule could be observed by videomicroscopy as the sudden swelling of the granule in the standard extracellular medium (Fig. 1 *A*).

Measurement of granule swelling

Swelling of secretory granules was measured using videomicroscopy and image analysis. Intact cells or isolated secretory granules were examined with a Zeiss IM35 inverted microscope equipped with Nomarski optics, using an oil immersion objective (Zeiss Planapo ×63, NA 1.4 or Zeiss Planapo ×100, NA 1.3; Zeiss, Oberkochen, FRG). Images were captured with a CCD video camera (model IKC 30M; Toshiba/Houston International Corp., Houston, TX, or model 4815–2000/0000; Cohu Inc., Electronics Division, San Diego, CA) and stored on a super-VHS video recorder (model BV-1000; Mitsubishi, Tokyo, Japan). The diameter of the granules was measured by using digital image analysis of sequential video frames which were displayed on a Sony video monitor (model PVM 1342Q) at a magnification of ~10,000×. Images were captured with a frame grabber (model DT285; Data Translation Inc., Natick, MA) installed on a microcomputer (Beltron 286) and analyzed using the Image-Pro software package (Media Cybernetics, Silverspring, MD). The granule cross-sectional area was measured by tracing the perimeter and counting the pixels contained within the boundary. Repeated measurements of the granule cross-sectional area gave an error of ~2%. Swelling is expressed as a percentage of the final swollen cross-sectional area obtained in standard extracellular saline, or as an expansion factor for the fractional increase in cross-sectional area upon lysis of the secretory granule. We have used the cross-sectional area, rather than the volume, because the cross-sectional area of a sphere is proportional to the surface area (surface area = 4 × cross-sectional area). It is the expansion in the surface area that would provide the force for pore expansion if the hypothesis examined in this paper is correct.

Cell capacitance measurements

The cell membrane capacitance was measured using the whole cell mode of the patch clamp technique. The pipette solution contained, in millimolar: 140 K-glutamate, 10 Hepes, 7 $MgCl_2$, 3 KOH, 0.2 ATP, 1 $CaCl_2$, 10 EGTA, 10 μM GTPγS (290 mmol/kg, pH 7.2). The Ca-EGTA buffer was prepared as described by Neher (1988). The free Ca^{2+} concentration in the pipette solution was 30 nM. The cell membrane capacitance was determined using a digital phase detector (Joshi and Fernandez, 1988), implemented on a system comprising a model 386/25 microcomputer (Compaq Computer Corp., Houston, TX) and an Indec (Sunnyvale, CA) data acquisition (IBX or IDA) interface. After applying a sinusoidal voltage (833 Hz, 54 mV peak to peak) to the stimulus input of the patch clamp amplifier (EPC-7, List Electronics, Darmstadt, FRG), the current was measured at two different angles from the stimulus, ϕ and $\phi - 90$. The phase detector was aligned so that one output (at $\phi - 90$) reflected the real part of the changes in the cell admittance (Re $[\Delta Y]$) and the second output reflected the imaginary part (Im $[\Delta Y]$). The phase was periodically adjusted by using the phase tracking technique (Fidler and Fernandez, 1989). A calibration signal for the capacitance trace was obtained by unbalancing the C-slow potentiometer of the compensation circuitry of the patch clamp amplifier by 100 fF. The capacitance of the cell membrane can be used to estimate the cell surface area by using a conversion factor of 10 fF/μm².

RESULTS

Swelling of isolated granules is inhibited by acidic histamine solutions

To test the hypothesis that granule swelling due to water movement through the fusion pore is the driving force for rapid pore expansion, we needed to establish conditions that inhibited the swelling of the granule matrix. It has recently been demonstrated that acidic histamine

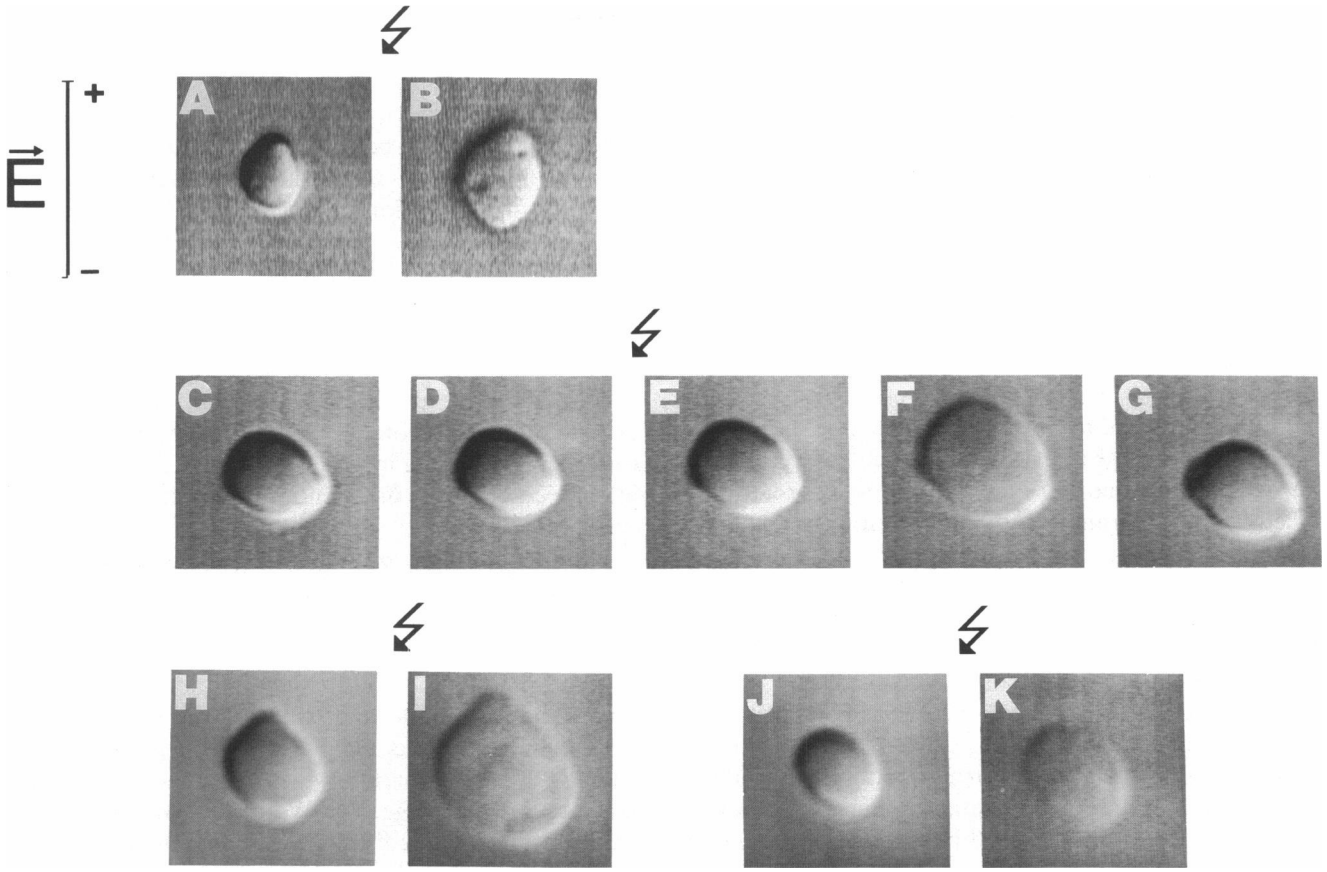

FIGURE 1 Swelling of isolated secretory granules from beige mouse mast cells is inhibited in an isotonic acidic histamine medium. (*A*) A Nomarski image of an intact single secretory granule in standard extracellular medium. (*B*) The same granule after being lysed by electroporation. Under these conditions the granules swell by a factor of 1.98 ± 0.07 (n = 5). (*C–G*) A sequence showing the effects of the acidic histamine medium on secretory granule swelling: (*C*) a granule in standard extracellular medium; (*D*) the same granule in isotonic acidic histamine medium; (*E*) the granule after electroporation in the acidic histamine; (*F*) the granule swells after being washed in standard extracellular medium, indicating that the cell membrane has been ruptured by the electroporation; (*G*) the acidic histamine medium can recondense the granule matrix. (*H* and *I*) another granule before *H* and after *I* being electroporated in standard extracellular medium supplemented with 2 M sucrose. (*J* and *K*) a granule before *J* and after *K* being electroporated in standard extracellular medium supplemented with 30% dextran.

solutions can recondense swollen proteoglycan from mast cell secretory granules (Curran and Brodwick, 1985; Villalon et al., 1990). Fig. 1, *A* and *B*, shows an isolated secretory granule from beige mouse mast cells before and after being lysed by electroporation in the standard extracellular medium. The electroporation technique briefly exposes the granule to a large electric field, which causes dielectric breakdown of the granule membrane near the anodic electrode and exposes the granule matrix to the medium outside the granule membrane. This local permeabilization mimics the action of the fusion pore in abruptly exposing the granule matrix to the extracellular medium (Fernandez, J. M., manuscript in preparation), which is why we used a Na^+ containing extracellular medium rather than a K^+ based intracellular medium. Upon lysis, the secretory granule swells and the cross-sectional area increases by a factor of 1.98 ± 0.07 (n = 5). A similar experiment performed in an isotonic histamine saline at pH 4.2 (see Methods) is depicted in the sequence in Fig. 1 *C–G*. The size of an intact granule, that is a granule with an unbroken membrane, was not altered on changing from the standard extracellular medium (Fig. 1 *C*) to the acidic histamine medium (Fig. 1 *D*). However, when the granule was lysed by electroporation in this histamine medium (Fig. 1 *E*) there was a slight swelling by a factor of 1.07, in sharp contrast to the rapid and extensive swelling observed in standard extracellular medium. It is clear that the granule membrane had been ruptured because subsequent exposure to standard extracellular medium caused an immediate swelling of the granule (Fig. 1 *F*). In the standard extracellular medium, the cross-

sectional area of the granule had increased by a factor of 1.81, but in acidic histamine medium the extent of swelling was reduced by 91%. As shown before (Curran and Brodwick, 1985; Villalon et al., 1990), readdition of the acidic histamine medium recondensed the granule (Fig. 1 *G*). In contrast to previous reports (Zimmerberg et al., 1987; Chandler et al., 1989), hyperosmotic solutions containing sucrose and dextran-containing solutions were not as effective in preventing the swelling of the secretory granule matrix (Fig. 1 *H–K*). The expansion factors were 2.26 ± 0.25 (n = 3) and 1.49 ± 0.16 (n = 5) for the swelling in media supplemented with 2 M sucrose and 30% dextran, respectively. Thus, the rate and extent of swelling of the secretory granule matrix that occurs when the interior of the granule is exposed to the external medium by electroporation is greatly reduced when the granule is bathed in an isotonic acidic histamine solution.

Exocytosis in acidic histamine saline

The degranulation of beige mouse mast cells was monitored by measuring the cell membrane capacitance. Stepwise capacitance increases caused by the fusion of individual secretory granules in cells bathed in standard and acidic histamine extracellular media are shown in Fig. 2. Exocytosis was induced by the nonhydrolyzable guanine nucleotide GTPγS, which was present in the patch pipette at 10 μM (Fernandez et al., 1984). Two examples of degranulation in standard and acidic histamine media are shown because there is some variability both in the latency before degranulation commences and in the rate of degranulation. Individual steps, representing the fusion of single–giant secretory granules, can be seen in the recordings. It is clear that guanine nucleotide stimulated exocytosis proceeded at a similar rate and to the same extent in the acidic histamine medium as occurred in normal extracellular medium. Reversible fusion events, fusions that end with closing of the fusion pore rather than pore expansion, occur under both conditions. The proportion of total fusion events ending in pore closure was 24% (n = 194) and 21% (n = 169) in the normal and histamine solutions, respectively.

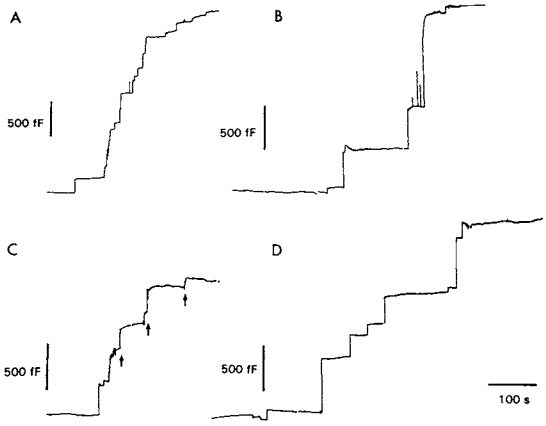

FIGURE 2 Acidic histamine extracellular medium does not prevent the fusion of the granules with the plasma membrane. The traces show stepwise increases in capacitance occurring in mast cells from the beige mouse that were completely degranulated by stimulating with intracellular guanine nucleotides. (*A* and *B*) Examples of complete degranulations in standard extracellular medium. Each step in capacitance represents the fusion of a single secretory granule with the plasma membrane. (*C* and *D*) Examples of complete degranulation in histamine external solution. The capacitance recording in *C* corresponds to the degranulation of the cell shown in Fig. 3 *A*, and the arrows represent the fusion of the granules whose swelling is shown in Fig. 3 *D*.

In contrast to the effects of the acidic histamine on secretory granule fusion, the swelling of the secretory granules was inhibited (Fig. 3). Fig. 3 *A* shows a mast cell bathed in acidic histamine medium in cell-attached patch clamp mode. Exocytosis was initiated by breaking into whole-cell mode and exposing the cytosol to the GTPγS in the pipette. Fig. 3 *B* shows the same cell after a complete degranulation, which is shown as the capacitance recording in Fig. 2 *C*. We can be sure that fusion has occurred because the ability of the standard extracellular medium to induce swelling of granules after the degranulation was complete shows that the granule lumen is connected to the extracellular space by a fusion pore (Fig. 3 *C*). The expansion factors for the increase in cross sectional areas were 1.72 ± 0.09 (n = 7) and 1.07 ± 0.03 (n = 9) for granules fused in the standard and acidic histamine extracellular media, respectively, indicating that the acidic histamine medium inhibits the extent of swelling by 90%, similar to the inhibition in the isolated granules (Fig. 1). Fig. 3 *D* shows the time courses of the swelling for three of the granules in the cell depicted in Fig. 3, *A–C*. For comparison, the time courses for the swelling of two granules from another cell that was degranulated in the standard extracellular saline are also shown. The fusion of the three granules in the acidic histamine medium correspond to the capacitance steps indicated by the arrows in Fig. 2 *C*. As well as reducing the extent of swelling, the rate of swelling is reduced by > 20-fold. Thus, when a mast cell is degranulated in acidic histamine medium the rate of granule swelling, as well as the extent, is greatly suppressed.

Fig. 4 (*A–F*) shows the time courses for the fusion of several individual secretory granules with the cell membrane. Both the real and imaginary components of the measured sinusoidal current are shown (see Methods). In the longer events, the rapid fluctuations in the real and imaginary traces, which are due to changes in the

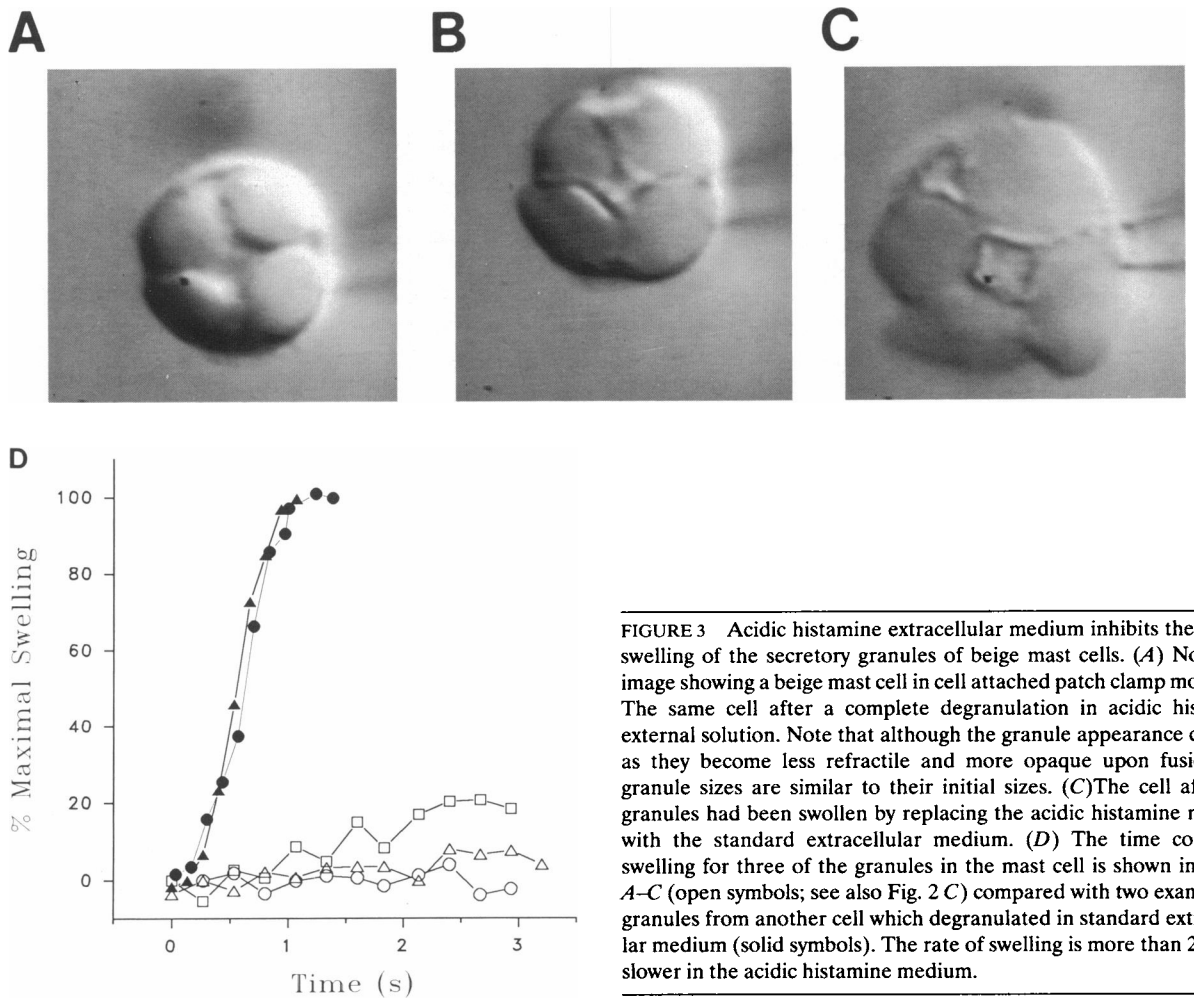

FIGURE 3 Acidic histamine extracellular medium inhibits the rate of swelling of the secretory granules of beige mast cells. (*A*) Nomarski image showing a beige mast cell in cell attached patch clamp mode. (*B*) The same cell after a complete degranulation in acidic histamine external solution. Note that although the granule appearance changes as they become less refractile and more opaque upon fusion, the granule sizes are similar to their initial sizes. (*C*)The cell after the granules had been swollen by replacing the acidic histamine medium with the standard extracellular medium. (*D*) The time course of swelling for three of the granules in the mast cell is shown in panels *A–C* (open symbols; see also Fig. 2 *C*) compared with two examples of granules from another cell which degranulated in standard extracellular medium (solid symbols). The rate of swelling is more than 20 times slower in the acidic histamine medium.

fusion pore conductance, can be seen (Fig. 4, *B, C, E, F*). As the fusion pore widens, the real component decreases sharply and the imaginary component simultaneously grows to a final value, the step increase being proportional to the membrane surface area of the secretory granule. Because the change in the real trace characteristically begins and ends with an abrupt transition (see Fig. 4, *A–F*), the period during which a projection can be seen on the real trace can be used as an estimate of the pore expansion time. This measure of the fusion pore expansion time has an advantage over previously used methods which, by measuring the time for the capacitance trace (imaginary) to reach half maximal or rise between 20 and 80% of the final step increase (Zimmerberg et al., 1987; Breckenridge and Almers, 1987*b*), can significantly underestimate the pore opening time (for example, see Fig. 4, *B, F*).

The examples of the fusion events shown in Fig. 4 include some with rapid expansion times (*A, D*) and also some with slower times (*B, C, E, F*). The mean pore expansion times were 400 ± 54 ms (n = 276) and 850 ± 190 ms (n = 135) in standard and acidic histamine extracellullar media, respectively. However, the mean expansion time is misleading because of the large variation in expansion times observed and their nonnormal distribution. The acidic histamine medium increases the proportion of events over 1 s in duration from 12% (33/276) to 17% (23/135). Several events (3/135) were over 10 s in length in the acidic histamine medium, whereas the longest event seen in the standard extracellular medium lasted just over 7 s. However, the majority of events are <1 s in length, comprising 88% (243/276) and 83% (112/135) of the total events in the standard and acidic histamine extracellular media, respectively. Fig. 5 shows the frequency distribution of pore expansion times that were <1 s in duration for experiments performed in normal and acidic histamine extracellular media. It is clear that there is no significant difference in the distribution of expansion times in the two media. Some of the fusion events are faster than the time resolution of our recordings (14 ms/point). However, expansion due to swelling is unlikely in these very fast

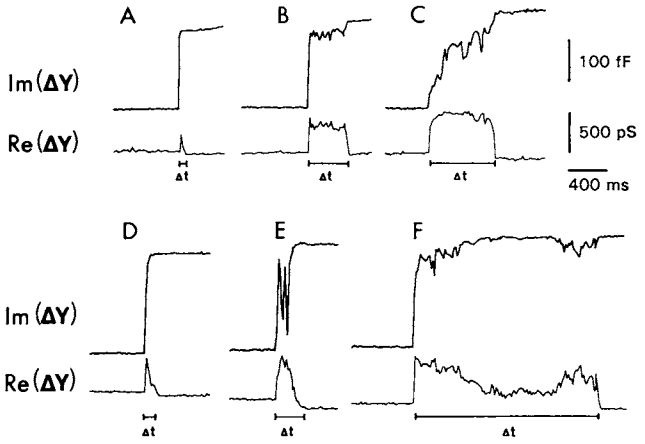

FIGURE 4 The time course of the fusion pore expansion is highly variable. (*A–C*) Three examples of the fusion of single secretory granules with the plasma membrane in cells bathed in standard extracellular medium. (*D–F*) Three examples in the acidic histamine medium. Each pair of traces shows the imaginary (Im, measured at a phase angle ϕ–90) and real (Re, measured at ϕ) parts of the admittances (ΔY) contributed by fused secretory granules. Im (ΔY) measures changes in membrane capacitance and Re (ΔY) measures resistive changes. Events as short as 14 ms (the time resolution of our recording system) and as long as 16 s were observed. The length of the projection on the real trace (Δt) can be used as a measure of pore expansion time.

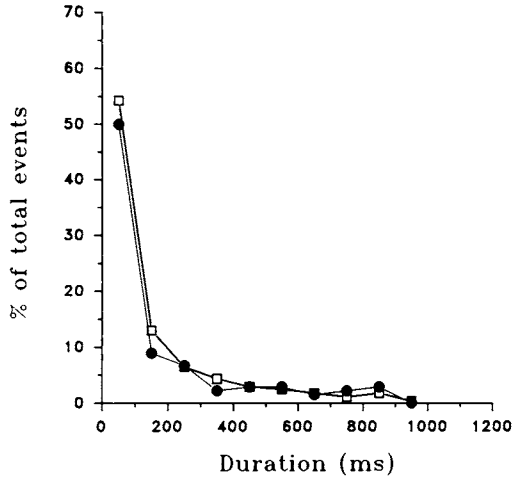

FIGURE 5 The lifetime of the fusion pore is not affected by the acidic histamine medium. The distribution of the pore duration times is shown for experiments in standard and acidic histamine extracellular media. The data were obtained by measuring the duration of the transient increase in the Re trace as shown in Fig. 4, and expressed as the fraction of lifetimes shorter than a specified duration. Only events up to 1,000 ms are shown (the bin width is 100 ms). A total of 276 events (34 cells) were measured in the standard extracellular medium, and the mean value was 400 ± 54 ms. A single exponential linear fit to the data gave a time constant of 205 ms. In the acidic histamine medium a total of 135 events were measured (19 cells), and the mean was 850 ± 190 ms (the fitted time constant is 210 ms). The events longer than 1 s in length comprise 12% (33/276) of the total events in the experiments in standard extracellular medium and 17% (23/135) in the experiments in acidic histamine extracellular medium. The first bin (0–100 ms) includes some events that are faster than the time resolution of our recordings: 8% (21/276) and 18% (24/135) of the total events in normal extracellular and acidic histamine media, respectively.

events because it has previously been shown that exocytotic fusion precedes granule swelling by tens of milliseconds in large mouse mast cells (Breckenridge and Almers, 1987*b*; Zimmerberg et al., 1987). Thus, in beige mouse mast cells granule swelling is not important for the large majority of the fusion events, although swelling may play a role in fusion pore expansion when the pore does not undergo the usual characteristic rapid expansion.

DISCUSSION

As a mast cell degranulates, the secretory granule contents are extruded from the cell through a fusion pore. When the fusion pore is formed, it provides a pathway for water to enter the proteoglycan matrix of the granule and swelling occurs. Various roles for the swelling have been proposed. First, it has been suggested that swelling of the granule matrix might be responsible for increasing the tension of the secretory granule membrane, which might be a necessary or important facilitating factor in the fusion event. Second, the swelling may provide the driving force for the rapid expansion of the fusion pore, without which the fusion pore may close. Finally, swelling of the granule can greatly facilitate dispersal of the granule contents. The latter function almost certainly is important, because without matrix swelling the biogenic amines, neurotransmitters and secretory peptides in secretory granules would be trapped and only slowly diffuse away. This clearly cannot be the case in rapid exocytosis as occurs in mast cells and neurosecretory cells.

The idea that the swelling might be important to the mechanism of exocytotic fusion derives from two discoveries. First, it was found that solutions of high osmolality could inhibit exocytosis of insulin containing granules from pancreatic β cells (Orci and Malaisse, 1980; Hermans and Henquin, 1986), chromaffin granules from adrenal medullary cells (Hampton and Holz, 1983; Pollard et al., 1984), and cortical granules from sea urchin eggs (Zimmerberg et al., 1985; Zimmerberg and Whitaker, 1985), suggesting the possibility that osmotic swelling of the secretory granule core was crucial to the mechanism of exocytosis. This view was made particu-

larly compelling by the other discovery, that osmotic forces could be used to induce fusion of phospholipid vesicles and bilayers in artificial fusion systems (Cohen et al., 1982; Finkelstein et al., 1986). Subsequently, however, it was found that some of the inhibitory effects of the hyperosmotic solutions were due to the formation of a granule-free zone beneath the plasma membrane that physically prevented the secretory granules from interacting with the plasma membrane (Merkle and Chandler, 1989; Chandler et al., 1989). Recently, several observations in mast cells, adrenal chromaffin cells, and sea urchin eggs have indicated that fusion of secretory granules with the plasma membrane, is not inhibited by hyperosmotic solutions (Holz and Senter, 1986; Whitaker and Zimmerberg, 1987; Zimmerberg et al., 1987; Breckenridge and Almers, 1987*b*). Furthermore, the fusion is seen to precede the swelling of the secretory granules (Breckenridge and Almers, 1987*b*; Zimmerberg et al., 1987). Thus, it seems that osmotic swelling of the secretory granules is not necessary for membrane fusion, although it should be noted that this conclusion has been criticized on the grounds that microswelling of the secretory granules might not be visible by light microscopy and could still occur in hyperosmotic solutions (Green, 1987; Lucy, 1989).

The possibility that swelling of the granule matrix, due to water entry through the fusion pore, plays a role in pore expansion has a more convincing case. First, dextran solutions were able to arrest pore widening in sea urchin eggs, apparently by inhibiting water entry into the granule (Chandler et al., 1989). Second, it has been suggested that pore dilation is slowed or prevented by hyperosmotic solutions in sea urchin eggs (Zimmerberg et al., 1987; Chandler et al., 1989). Third, the fusion of secretory granules with the plasma membrane is a reversible process; this cannot be the case if the granules have already swollen, suggesting that granule swelling might be the event that pushes exocytosis to completion. Finally, some property of the exocytotic apparatus must be responsible for the rapid pore expansion in exocytosis, because, in contrast to the exocytotic fusion pore, the fusion pore that is induced between two plasma membranes by influenza virus hemagglutinin expands at a rate several orders of magnitude slower (Spruce et al., 1989). In view of these experiments, we considered it likely that inhibition of granule swelling would have two effects. First, the time which the fusion pore existed in the flicker state before final irreversible expansion would be increased, perhaps indefinitely, and second, the proportion of events in which the fusion pore collapsed would be increased.

As a first step, we needed to establish conditions that could prevent swelling of the secretory granule. The proteoglycan matrix contained in mast cell secretory granules undergoes a two to threefold increase in volume when exposed to standard Na^+ containing extracellular medium by the fusion pore opening. Experimentally, swelling of the granule matrix can be induced in two ways: in isolated mast cell granules in vitro by electroporation or in intact mast cells by stimulating exocytosis (see Methods). Because the secreted granule matrix can be recondensed by acidic histamine (Curran and Brodwick, 1985; Villalon et al., 1990), we used an isotonic histamine medium at pH 4.2 to inhibit swelling of the matrix proteoglycan. The high histamine concentration and acid pH of this solution mimics the environment thought to occur in intact secretory granules, a proteoglycan matrix condensed with divalent histamine ions (Alter and Schwartz, 1989). The acidic histamine medium inhibits the extent of the granule matrix swelling by an order of magnitude in both electroporated isolated secretory granules (Fig. 1) and in exocytosing mast cells (Fig. 3). Moreover, the rate of swelling of the granule after fusion is reduced by >95%, providing a simple experimental method for perturbing granule swelling and studying the effect on exocytosis.

The ability of mast cells to undergo guanine nucleotide stimulated increases in cell membrane capacitance is unimpaired when the cells are bathed in the acidic histamine medium (Fig. 2). The capacitance increase can be seen as discrete stepwise increases due to fusion of single-secretory granules. The fusion of three of the secretory granules in the beige mouse mast cell shown in Fig. 3, *A–C*, was observed as a sudden change in the refractive properties of the granule and coincided with the capacitance steps indicated by the arrows in Fig. 2 C. Individual fusion events were analyzed to see if the acidic histamine medium, which had such a marked effect on the swelling of the granules, had any effect on the pore expansion time. The majority of fusion pores expanded in <1 s. The relative frequencies of the pore open times for these events were identical in the standard extracellular and acidic histamine media (Fig. 5). However, the acidic histamine medium increased the proportion of events lasting longer than 1 s from 12% (33/276) to 17% (23/135) which resulted in a significant increase in the mean pore expansion time from 400 ± 54 (n = 276) to 850 ± 190 (n = 135). Therefore, for these longer events, swelling of the secretory granule does appear to play a role in driving pore expansion and probably provides a fail-safe mechanism. On the other hand, in the majority (~90%) of the fusion events the rate of pore expansion is unchanged under conditions where the rate of granule swelling differs by >20-fold, indicating that water entry through the fusion pore is not the normal driving force for pore expansion in mast cells of the beige mouse. These experiments do not exclude the possibility that the granule swelling is induced by a

mechanism that does not utilize the fusion pore for water entry. The opening of some yet to be defined ion channels in the granule membrane could be involved in such a process. However, because secretory granule swelling is not seen before fusion (Breckenridge and Almers, 1987; Zimmerberg et al., 1987), the swelling would have to be spatially localized in such a way that it was not detectable using light microscopy as has been previously suggested (Green, 1987; Lucy, 1989). Alternatively, an interaction of the cytoskeleton with the membrane forming the fusion pore could provide the force to drive pore expansion.

Recent observations of the kinetics of reversible fusion events have indicated that there is membrane transfer from the plasma membrane to the secretory granule while they are connected by the fusion pore (Monck et al., 1990). It was proposed that the membrane transfer occurs because the secretory granule membrane is under tension. Because many of the protocols that have been used to induce fusion of phospholipid vesicles and bilayers, which include osmotic and hydrostatic force, divalent cations, temperature, electromechanical stress, and membrane "depletion," cause an increase in membrane tension, it was suggested that the secretory granule membrane tension plays a critical role in the mechanism of exocytotic fusion (Zimmermann, 1986; Ohki, 1987; Holz, 1986; Helm et al., 1989; Finkelstein et al., 1986). Tension in the secretory granule membrane is also a candidate for the force that drives fusion pore expansion. An obvious candidate for the mechanism for generating this tension is swelling of the secretory granule core. However, swelling of the granule matrix as a consequence of water influx through the fusion pore is unlikely to be the mechanism because the rate of membrane transfer through the fusion pore (assumed to be a measure of the tension difference between the two membranes) was not altered in experiments in which the cells were bathed in acidic histamine media (Monck et al., 1990). Other possible mechanisms for generating the tension include granule swelling due to transport mechanisms present in the secretory granule membrane and interactions of the secretory granule with the cytoskeleton. Thus, it seems possible that the mechanism that generates the tension in the secretory granule membrane could serve the dual purpose of promoting fusion and driving the rapid expansion of the fusion pore necessary for rapid release of secretory granule contents.

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